The temporal expression of fast skeletal muscle myosin heavy chain isoforms related to muscle growth in poultry

Monica Miller

June 2010

With the help of:
Dr. Macdonald Wick
John Mark Reddish

Abstract

In all animals, fast skeletal muscle grows during development by completing a series of temporal expression of muscle proteins and myosin heavy chain (MyHC) isoforms. During development, the muscle is populated with ventricular MyHC, then by embryonic 1, 2, 3, (Cemb1, Cemb2, Cemb3), followed by neonatal (Cneo) and finally an adult (Cadult) MyHC isoform. The functional roles of the MyHC isoforms are unknown. In order to identify the roles of MyHC isoforms during development, we investigated MyHC expression in broiler and layer chickens at the RNA level. Total RNA was extracted from *Pectoralis major* (PM) muscle samples taken from broiler chickens, layer chickens and quail and run with isoform-specific primers in semi-quantitative reverse transcriptase-polymerase chain reactions (RT-PCR). The broiler chickens were found to start developing the adult isoform sooner, chronologically, than the layers. The neonatal isoform concentration was also showed to peak sooner in the broilers than the layers. The quail samples were run with the embryonic isoforms and only have preliminary results showing differences between three different strains and their isoform transition rates. This information can be used as a base to develop a method to study muscle development at similar cellular times for comparative studies of temporal events.

Introduction

The poultry broiler industry is based primarily on muscle yield in the *Pectoralis major* (PM). In order to increase production and profit, broiler chickens have been selected to grow larger breast muscles. We have genetically selected broilers to grow the PM larger at a faster rate (1, 2, 3), but not any of the other muscles in the body. It is currently unknown what the relationship between the temporal expression of the

developmental myosin heavy chain (MyHC) isoforms is related to muscle growth. The sequence of expression is crucial to development because it was determined that injured muscle undergoes the same temporal and spatial pattern during regeneration (1, 4, 5). Therefore, understanding the expression and development of MyHC isoforms can potentially lead to greater understanding of myopathy recovery. Past studies search has been done to determine the temporal expression of all of the isoforms in chicken (2, 3, 4, 6, 7). This work has demonstrated that the PM of chickens is comprised, at least partially, of the neonatal isoform from the day of hatching to approximately day 35, and the adult isoform from day 10 posthatch. The ventricular and embryonic isoforms were not shown to be active after day 10 posthatch. That study only used layer chicken lines, however, and simply determined the presence of the isoforms. In this project, the temporal expression as related to muscle development is being further studied by comparing the relative concentrations of each isoform between the layer and faster growing broiler lines. Corresponding points in physiological development, rather than chronological age, can further solidify the viability of developmental checkpoints as references for comparative studies.

The hypothesis is that the regulation of the expression of the MyHC isoforms is related to the mechanisms of muscle growth, with an application in how it can be altered through genetic selection. Thus, the temporal expression of the MyHC is related to temporal muscle development and repair mechanisms. The objective of this project ultimately was aimed at developing a method to study muscle development at similar cellular times as staging molecules for comparative studies of the temporal events in muscle development.

Methods

Muscle Samples

Samples of PM were harvested from broilers and single-comb white leghorn layers (SCWL) at the ages 1 day (the day of hatching), 5 days, 11 days and 22 days. The PM samples were also harvested from heavy weight (HW), light weight (LW) and random bred control (RBC) strain quail on embryonic days (ED) 8 and 10. The RNA was extracted using TRIzol and the corresponding protocol (Molecular Research Center, Inc.) and stored in 50:50 formamide/water at -80°C. The integrity of the samples was tested by running on 1.5% Tris/Borate/Ethylenediaminetetraacetic acid (TBE) agarose electrophoresis gels at 100 volts. These gels were examined and photographed under ultraviolet light for the presence of bands representing the 18S and 28S subunits of the ribosomal RNA molecule. A 1000 base pair ladder was run with the samples for fragment size comparison.

RT-PCR

The RNA samples were mixed with One-Step standard RT-PCR (Invitrogen) forward and reverse primers (Operon) specific to each isoform (8), as well as primers for glyceraldehydes 3-phosphate dehydrogenase (GAP-DH) in separate reactions as a housekeeping gene, due to its presence in all living muscle cells and for future use in band quantification as a reference point. The RNA from the muscle samples was denatured at 94˚C and then annealed at 55˚C for 35 cycles (Invitrogen protocol). The primer sequences are as follows:

Table 1. RT-PCR primer sequences and expected product sizes.

Primer Name	Forward Primer Sequence[1]	Reverse Primer Sequence[1]	Product Size[2]
Cemb1	GTCCAAGAAGACACGAAGACGTCGGACC	AAGCGCAGCGCTGAAGCCGTGAAGGG	623
Cemb2	TCACACCTCCTCGACTTGAGAGA	AAGCGCAGCGCTGAAGCCGTGAAGGG	392
Cemb3	TGTGACGGACACACAAAGAG	AAGCGCAGCGCTGAAGCCGTGAAGGG	590
Cneo	CTCCATACCCAGAACACC	AAGCGCAGCGCTGAAGCCGTGAAGGG	378
Cadult	GACCTGAACGAAATGGAG	AAGCGCAGCGCTGAAGCCGTGAAGGG	664
GAP-DH	GAGGGTAGTGAAGGCTGCTG	CCACAACACGGTTGCTGTAT	199

[1]Sequences are listed 5' to 3'.

[2]Measured in base pairs.

Electrophoresis

Products of the RT-PCR (6μL) were mixed with loading dye (3μL) and run on 2% Tris/Acetate/Ethylenediaminatatraacetic acid (TAE) agarose electrophoresis gels at 100 volts. A 100 base pair ladder was run with the samples for fragment molecular weight measurement. Fragment sizes generated by each primer can be seen in Table 1. These fragments were identified by comparing the distance they traveled in the gel compared to the distance traveled by the ladder. The various sizes of the fragments also allows for distinguishing one from another. They were observed and photographed under ultraviolet light.

Results

The electrophoresis band staining intensity, observed visually, showed differences in concentrations in the broilers and layers, between the various ages. As seen in Figures 1 and 2, the amount of mRNA transcript found in broiler and layer PM increases with the age of the samples.

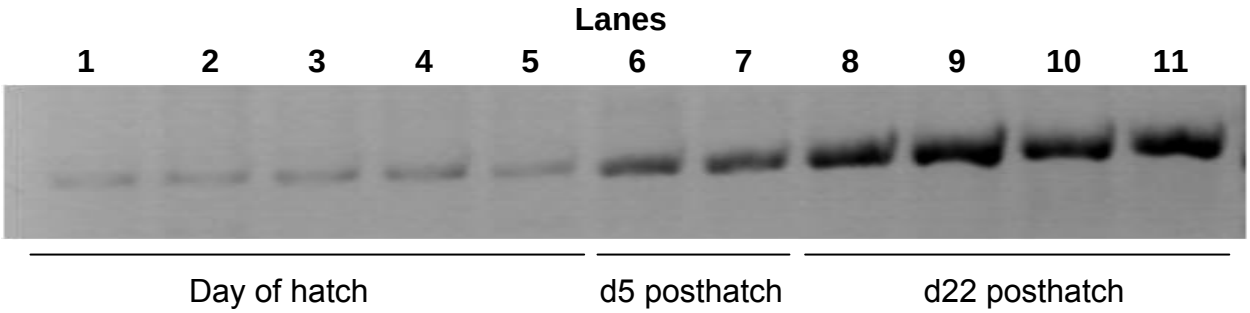

Figure 1. Broiler chicken PM RT-PCR product; run with adult isoform primer.
Lanes 1-5: samples from the day of hatching (1 day posthatch).
Lanes 6-7: samples 5 days posthatch.
Lanes 8-11: samples 22 days posthatch

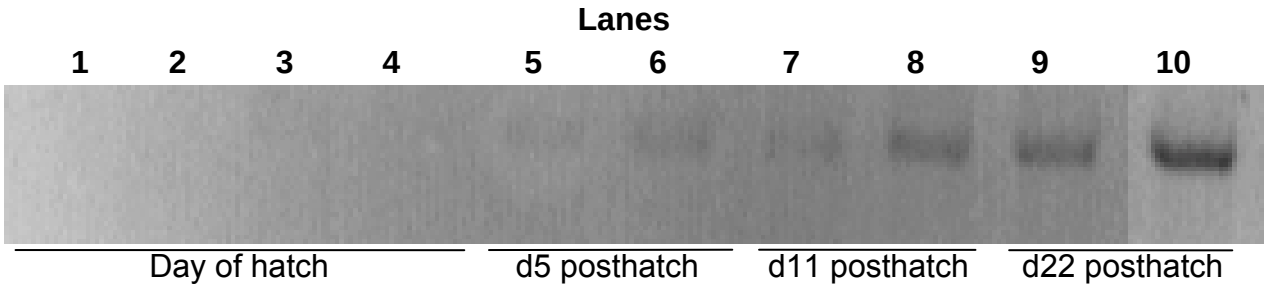

Figure 2. Layer chicken PM RT-PCR product; run with adult isoform primer.
Lanes 1-4: samples from the day of hatching (day 1).
Lanes 5-6: samples from 5 days posthatch.
Lanes 7-8: samples from 11 days posthatch.
Lanes 9-10: samples from 22 days posthatch.

On the other hand, the neonatal isoform, while showing variation between the different ages, appears to peak around day 5 in the broilers and between day 5 and day 11 in the layers (Figures 3 and 4).

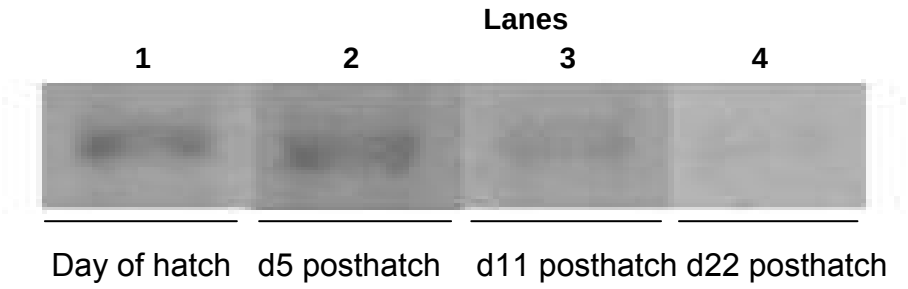

Figure 3: Broiler chicken PM RT-PCR product; run with neonatal isoform primer.
Lane 1: 1 day posthatch.
Lane 2: 5 days posthatch.
Lane 3: 11 days posthatch.
Lane 4: 22 days posthatch.

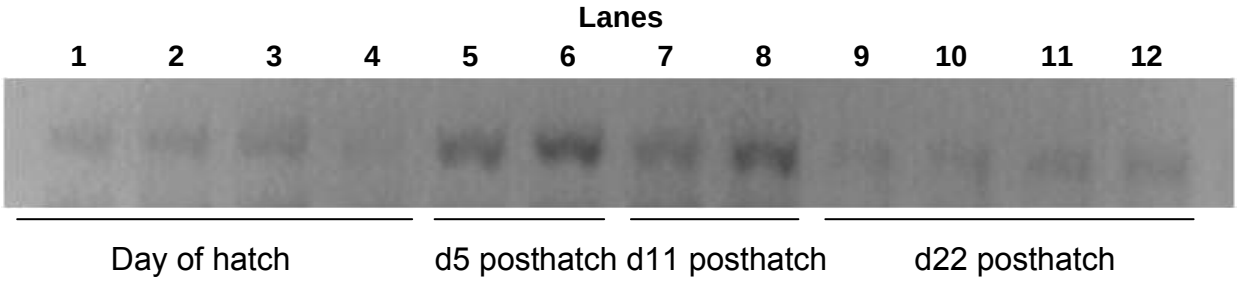

Figure 4: Layer PM RT-PCR product; run with neonatal isoform primer.
Lanes 1-4: 1 day posthatch.
Lanes 5-6: 5 days posthatch.
Lanes 7-8: 11 days posthatch.
Lanes 9-12: 22 days posthatch.

Quail samples were run with the embryonic isoform primers, based on prehatch age. Depending on the age (day 8 or day 10) and the primer (Cemb1, 2, or 3), some samples generated RT-PCR product and some did not. Those that did produce product were: ED8 HW Cemb1, ED8 LW Cemb1, ED10 HW Cemb1, ED10 RBC Cemb1, ED10 LW Cemb1, ED8 LW Cemb2, ED10 HW Cemb2, ED10 RBC Cemb2, ED10 LW Cemb2, ED8 HW Cemb3, ED10 HW Cemb3, ED10 RBC Cemb3, ED10 LW Cemb3 (Figure 5).

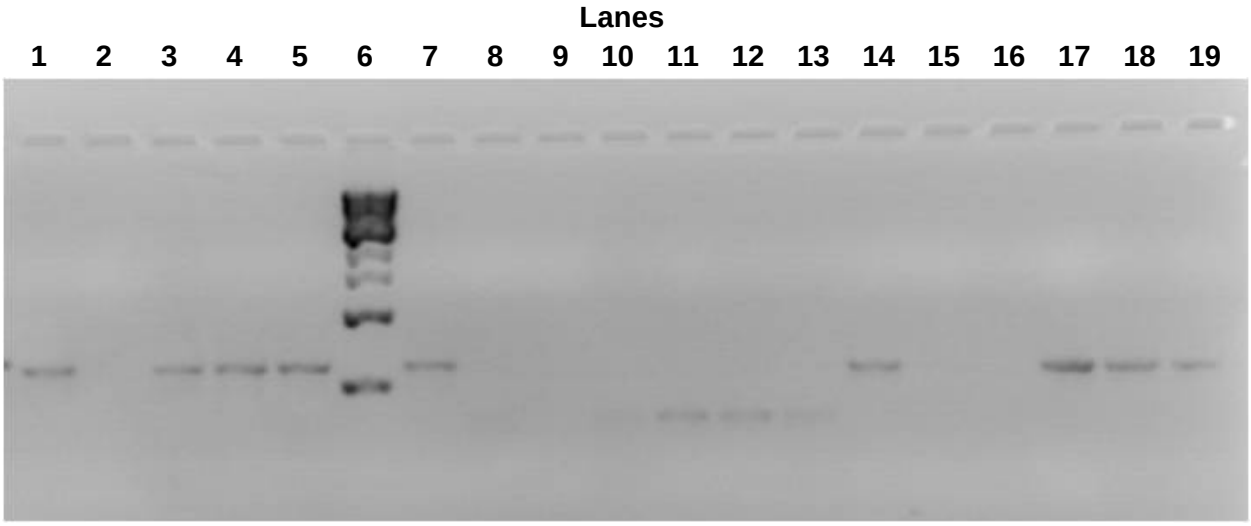

Figure 5: Quail (PM RT-PCR product; run with embryonic 1, embryonic 2, and embryonic 3 primers.
Lanes 1-3: embryonic day (ED) 8, embryonic 1 primer (Cemb1), heavy weight (HW), random bred control (RBC) and light weight (LW) samples, respectively.
Lanes 4-5: ED10, Cemb1 primer, HW and RBC samples, respectively.
Lane 6: 1 kb ladder
Lane 7: ED10, Cemb1 primer, LW sample.
Lanes 8-10: ED8, embryonic 2 primer (Cemb2), HW, RBC, LW samples, respectively.
Lanes 11-13: ED10, Cemb2, HW, RBC, LW samples, respectively.
Lanes 14-16: ED8, embryonic 3 primer (Cemb3), HW, RBC, LW samples, respectively.
Lanes 17-19: ED10, Cemb3, HW, RBC, LW samples, respectively.

Discussion

Although the mechanisms of the isoforms is still not known, this project has built upon previous studies demonstrating the temporal expression of MyHC isoforms. The neonatal and adult isoforms have been identified in broilers and layers, and preliminary work has been done with the embryonic isoforms in quail. The neonatal isoform has was present in chickens from hatching to day 22. The adult isoform was also found throughout that time period in broilers, but only from day 5 posthatch on in the layers. This suggests that the broilers are developing the PM faster than the layers, and that by day 5 posthatch, physiologically the broilers are 5 days older than the layers. The findings with the adult isoform are in contrast to previously published studies that stated

the muscle begins to be comprised of adult MyHC around the age of 10 days (4). These investigator's previous research (4), which was completed 13 years ago, focused solely on layers. Therefore, differences in results from the project completed here were likely due to the genetic selection of the broilers and overall genetic selection increasing the rate of development of the layers.

The concentration of adult isoform in the chicken PM follows a gradual increase over 22 days in broilers and layers (Figures 1 and 2). It was not determined whether this increase is linear or exponential,and requires further study. In addition, a comparison of the MyHC isoform expression in the broilers and layers will be undertaken to determine at which ages they have reached and equal physiological developmental stage.

Broilers and layers had differing amounts of neonatal MyHC isoform present (Figures 2 and 3). The band that was most intense in the broilers appeared at day 5, while in the layers it ranged from day 5 to day 11 posthatch. This suggests that the layer breast muscle contained a high concentration of neonatal MyHC later, chronologically, than did the broilers. As was seen with the adult isoform results (Figures 1 and 2), consequently, the layers likely had a more immature muscle at the same age. The crucial information to be determined with this data is the percentage of the muscle that is neonatal and adult. Doing so will help to figure out the timing and speed of the transition between the types of MyHC isoforms, therefore creating part of the timeline for temporal expression. With the reactions that have already been run, this can be accomplished by quantifiying the intensity of the bands and comparing relative percentages of the whole (the total MyHC present in all of the bands combined).

The final work completed thus far has just begun. To date, no data has been published attempting to identify the MyHC isoforms in quail. The three breeds utilized were a random bred control (RBC), a strain bred for heavy muscling ("heavy weight," or HW) and a strain bred for less muscling ("light weight," or LW). The samples collected were from embryonic days (ED) 8 and 10, and the RT-PCR products that appeared with each strain and primer are shown in Figure 5. The HW line contained Cemb1 and Cemb3 at ED8, and all three isoforms were present at ED10. The RBC line only contained any of the isoforms at ED10. The LW line only did not produce product in ED8 with Cemb3. How these lines overlap can be seen in Figure 6. The conclusions that can be drawn from this data are limited; it would appear that the LW line is more immature than the HW line, based on the fact that it does not have Cemb3 present at ED8. However, the fact that the HW line does not have Cemb2 present at ED8, but does have Cemb1 and Cemb3, states that more tests need to be run, as this data does not support the current knowledge of the order that the isoforms develop in. It can be inferred, however, that the RBC line is less developed than either the HW or LW lines at the same chronological age. Using the primers created for chicken, we were able to produce RT-PCR product of appropriate sizes with the embryonic (1, 2 and 3) primers. This suggests that the nucleic acid coding for the isoforms are highly conserved between species. The data gathered also showed potential differences between breeds, similar to the chronological and physiological differences between the broiler and layer.

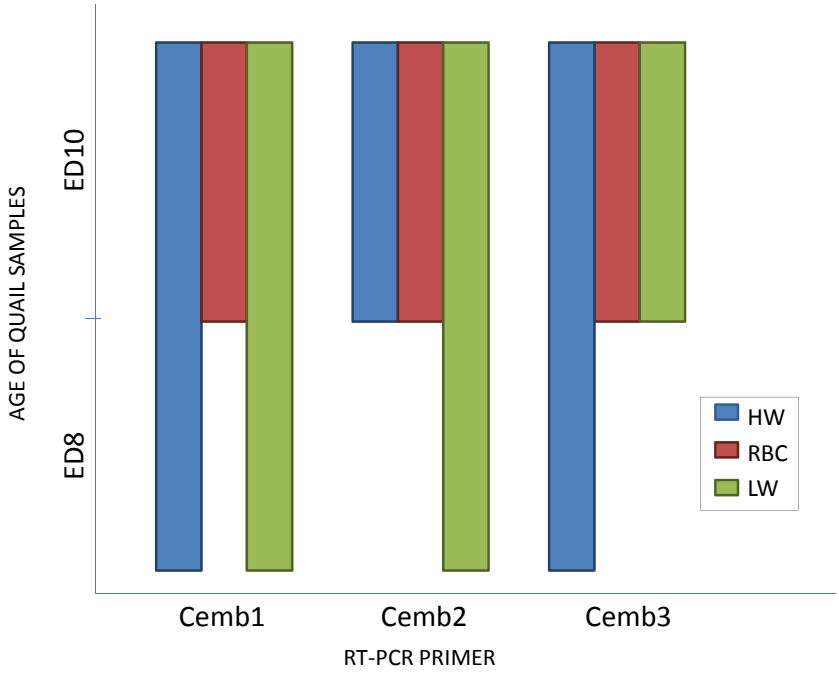

Figure 6. Presence of embryonic RT-PCR primers run with quail PM samples. Representation of the presence of RT-PCR product when quail samples of three lines [heavy weight (HW), random bred control (RBC) and light weight (LW)] were run with MyHC embryonic 1, 2 and 3 primers. A bar in the graph indicates the Cemb RT-PCR product was found when run with said sample at said age.

Conclusion

We have begun to develop a method, using the temporal expression of MyHC isoforms as staging molecules, to study muscle development at similar cellular times for comparative studies of the temporal events in muscle development.

The results of this research have implications in both applied agricultural and basic muscle biology sciences. Understanding the ways we have modified poultry development through selective breeding can give insight to making precise genetic selection for genes resulting in other quantitative traits of economic importance in animals. This research has begun to produce results demonstrating a molecular timeline for the cellular changes during muscle development.

In this study, we produced RT-PCR primers for five of the six development fast skeletal muscle MyHC isoforms. Thus far, the adult and neonatal isoforms demonstrated differing amounts of developmental MyHC isoform concentration at different posthatch ages of chickens. These preliminary studies have shown that the embryonic isoforms are also found in quail samples. Evidence of the embryonic MyHC isoforms at the RNA levels in quail have never been published prior to this project. In the future isoforms present will need to be quantified in order to determine a more accurate developmental timeline.

Literature Cited

1) Maruyama K., Kanemaki N., Potts W. and J.D. May. 1993. Body and muscle growth of domestic turkeys (Meleagris gallopavo) and expression of myosin heavy chain isoforms in breast muscle. Growth Dev Aging, 57 (1): 31-43.
2) Reddish J.M, Wick M., St. Pierre N.R. and M.S. Lilburn. 2005. Analysis of myosin isoform transitions during growth and development in diverse chicken genotypes. Poult Sci, 84: 1729-34.
3) Wick M., Reddish J.M., St. Pierre N.R. and M.S. Lilburn. 2003. Biochemical analysis of muscles from poultry bred for rapid growth. Poult Sci, 82: 1980-84.
4) Tidyman W.E., Moore L.A. and E. Bandman. 1997. Expression of fast myosin heavy chain transcripts in developing and dystrophic chicken skeletal muscle. Devel Dyn, 208.4: 491-504.
5) d'Albis A. and R. Couteaux. 1988. Regeneration after cardiotoxin injury of innervated and denervated slow and fast muscles of mammals. Euro J Biochem, 174: 103-10.
6) Maruyama K and N. Kanemaki. 1991. Myosin isoform expression in skeletal muscles of turkeys at various ages. Poult Sci, 70 (8): 1748-57.
7) Taylor L.D. and E. Bandman. 1989. Distribution of fast myosin heavy chain isoforms in thick filaments of developing chicken pectoral muscle. J Cell Bio, 108: 533-542.
8) Moore L.A., Arrizubieta M.J., Tidyman W.E., Herman L.A. and E. Bandman. 1992. Analysis of the chicken fast myosin heavy chain family: Localization of isoform-specific antibody epitopes and regions of divergence. J Mol Bio, 25.4: 1143-51.